Enzymatic Defenses of Certain Snails Against Metal Ions

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INTRODUCTION

Little study has been given to the defenses of snails against heavy metal ions even though it has long been known on a pragmatic basis that aquatic mollusks are highly sensitive to copper ions. Furthermore, copper ions have been used with some success against aquatic snails as a means of control. Since some snails appear to be more resistant to copper used as a control a study to assay possible defenses in one of these more highly resistant species should be of some general interest to snail physiology.

The shell is the first line of snail defense which protects the greater part of the otherwise poorly covered body from membrane-contact with the external environment. If the snail is an operculum-bearing prosobranch the ability to isolate itself from the surrounding environment is increased. Any ability to metabolize anaerobically further extends the period during which the snail can remain self-imprisoned during ionic crises in the surrounding environment and thus enhances the probability of ultimate survival.

During a previous study using *Oncomelania formosana* (Pilsbry & Hirase, 1905) as the experimental animal it was determined (Winkler & Chi, 1964) that certain cells free in the hemocoel picked up copper during chronic poisoning by very low levels of copper added to the environmental bath provided for these amphibious prosobranch snails. During the study it was further observed that snail squashes exposed to copper solution turned blue. Immersing live de-shelled snails in copper solutions similarly caused light blue areas to appear on the surface of the mantle, midbody and even on areas of the foot and digestive gland. The possibility that this represented a defensive reaction of protective value to the snail served as the take-off point for the present study.

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MATERIALS and METHODS

Snails used in this study were obtained from stock dishes of *Oncomelania formosana* (Pilsbry & Hirase, 1905) reared and maintained in our laboratory as previously described (Wagner & Chi, 1959). In order to remove the protection provided by the shell and operculum and to produce a degree of uniformity in the results all snails were de-shelled prior to use. The living snails were placed in 1% copper sulfate for the empirically chosen time periods of 3, 10, and 30 minutes, and 1, 3, 5 and 7 hours. This concentration was chosen to speed results since de-shelled snails do not live more than 24 hours. Immediately after exposure snails were killed and fixed in acetone at deep-freeze temperatures after which they were allowed to return to room temperature for storage. Snails destined for histochemical study were removed from acetone to two changes of tertiary butanol for a total of two hours, and then to benzene with one change for a total time of 15 minutes. From the last benzene the snails were transferred to about 5 ml of molten paraffin for 2½ hours, followed by conventional embedding procedures. This abbreviated procedure produced very adequate results with the small amount of tissue found in these snails. After serial sectioning and subsequent mounting on glass slides the sections were either left unstained for direct observation and chemical study of the blue colored objects, or were treated with the rubeanic acid histochemical reagent for demonstration of micro amounts of copper using a modified method similar to that of Uzman (1956). Eosin was used as counterstain. The carbonic anhydrase method of Gomori (1952) employing cobalt was used on de-shelled acetone-fixed snails for confirmation of the presence of this enzyme. Snails were killed in subzero acetone, then

treated with the reagents, after which they were sectioned and mounted. Parallel procedures using copper were carried out for comparative purposes.

Quantitative determination of the rate of concentration of copper in the snail was made by exposing 6 de-shelled snails for each of the above mentioned time intervals. These were then rinsed and stored in cold acetone. After storage the acetone was removed with a minimum of ½ hour air evaporation. A modified form of the procedure of Gubler *et al.* (1952) was used for spectrophotometric determination of copper. Individual snails, after being weighed to 4 decimal points on a Mettler H-15 balance, were placed in the courvette, after which 10 drops of 2N HCl were added and left to stand for 20 minutes. The solution was then made slightly alkaline with 4 drops of 1:3 ammonium hydroxide and the level was brought to a total of 3.2 ml with de-ionized water. After setting the 100% transmittance reading of the Spectronic 20 with this solution, 3 drops of diethyldithiocarbonate in 0.1% solution was added and the resultant reading was recorded from the photometer at 440 μ. If this transmittance reading was below 10 on the scale, an additional 3.2 ml of de-ionized distilled water was added and a second more accurate reading was obtained by halving the subsequent reading as indicated on the spectrophotometer. The copper present was then read in γ of copper from a curve previously prepared, using the same procedure on graded dilutions made from a standard copper solution.The addition of sodium pyrophosphate and citrate recommended by the original writer was omitted, since the control snails gave a uniform reading of under one γ of copper-equivalent of the combined iron and copper impurities. The results in γ per snail were finally converted to γ of copper per milligram of snail tissue and plotted against time (Figure 1 a).

In order to determine the rate of copper concentration by carbonic anhydrase when the protective, living processes of the snail were removed, snails were killed by immersion in subzero acetone which did not deactivate the enzyme systems, and were then stored at deepfreeze temperatures for at least 12 hours. They were then exposed to the copper bath for the same time intervals as used in the previous experiments. After removal each was stored in acetone until it was quantitated by the procedure previously described (Figure 1 b).

RESULTS

The most significant observation noted on the sectioned material was the presence of large numbers of birds-egg-blue crystals which were effervescent when weak hydrochloric acid was introduced under the cover slip. This,

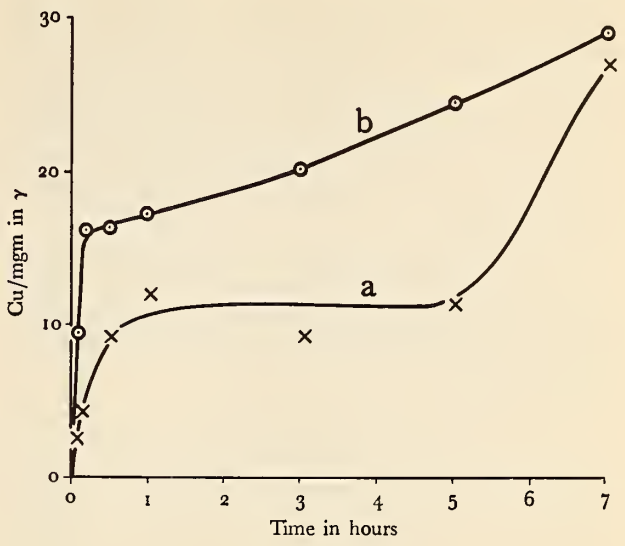

Figure 1

Curves showing rate of copper uptake by *Oncomelania formosana*. Curve *a* is that of de-shelled but living snails; *b* is the result when the snails were killed by storage in cold acetone – a procedure which but slightly reduced the enzyme activity. Each point represents averages for 6 and 4 snails on the two curves respectively. The copper levels of individual living snails of curve *a* were wider in range than those of the dead snails *b*, especially in the flat portion of the curve.

combined with their color, identified them as $CuCO_3$. These crystals were found covering the surfaces of the mantle, central body wall and, to a more limited extent, the foot and digestive gland. Furthermore, they also occurred in the peripheral tissues in specimens exposed for the longer time periods. Associated with these crystals were numbers of the free, spherical cells described in the previous paper; however, in these unstained sections many showed the same light blue color as the crystal formations. In addition, careful examination revealed the presence of one to many crystals contained in most of those showing the blue color. The Gomori (1953) carbonic anhydrase activity test established these cells along with certain areas of the body wall, specifically those covering loose connective tissue, as the principal sites of activity of that enzyme.

The copper penetration observed in the rubeanic acid-stained sections of snails exposed only briefly was predominantly in the midbody region with very little in the foot or in the digestive gland despite the very thin walls of the latter. Under limited exposure periods very few copper carbonate crystals were observed. Few of the protective cells (p-cells) were noted peripherally but these increased in number with time as did also the formation of copper carbonate.

The chief loci of copper carbonate as well as of clumped p-cells were in loose connective tissue, usually immediately below the thin outer walls. It was difficult to separate the crystal formation from the clumps of these p-cells. Particularly was this true under the high sensitivity of the rubeanic acid test which tended to blur the clumps of cells. Unstained slides were distinctly superior under these conditions.

As time of exposure progressed concentration rose in the peripheral connective tissue of the mid-body area. Only in exposures above 3 hours was there considerable invasion in other than peripheral tissues of the foot and digestive gland.

The extent of the immobilization of soluble copper and its concentration into insoluble copper carbonate was studied using snails killed in subzero acetone in order to retain enzyme activity. When the amount of copper fixed in each snail was quantitated, averaged and graphed (Figure 1 b) it was observed to rise sharply to 16γ per mgm in those with 10 minutes exposure. Thereafter the curve sharply leveled, then continued its rise at a gentler slope terminating at 27γ in snails of 7 hour exposure. When the living processes were superimposed by not killing the snails till after copper exposure (Figure 1 a), the curve rose considerably less precipitously to a little over 10γ of copper per mgm during the first half hour. This was followed by 4½ hours in which little change was observed but in which considerably more variability occurred between individuals of the same exposure increment. The resistance of the living process appeared to break down somewhere between 5 and 7 hours, allowing a rapid rise to approximately the same level as that of the snails exposed after having been killed.

DISCUSSION AND CONCLUSIONS

The observed formation of insoluble $CuCO_3$ seems to be a part of the snails' defenses rather than only a byproduct of shell formation since the centers of carbonic anhydrase activity revealed by the Gomori test were principally the p-cells which were widely spread throughout the snail body. Copper used in place of cobalt in a parallel procedure also revealed one factor that appears in distinct contrast between the two ions. While cobalt localized itself in the sites of carbonic acid activity with very little attaching to the tissues, copper attached itself extensively on all tissues This is not the result of greater sensitivity of the test as the same "sulfide procedure" for detecting the presence of cobalt was used for the copper in this procedure. One exception to the above statement was the strong affinity for the primary reproductive organ evident by heavy differential staining of the latter tissue when using the cobalt procedure.

The p-cells exist in various sizes and may be observed thus in the various clumps where they occur. When iron was substituted for the copper, the p-cells were not masked by staining of surrounding areas. It was then possible to locate what appeared to be the sites of origin of the p-cells in pockets of the loose connective tissue. In these pockets large numbers of miniature p-cells of homogeneous sizes were observed. These were interpreted to be developing p-cells.

When iron or calcium was substituted for copper, the results were less definitive. In both cases the cells gave the stain characteristic of the ion involved, but crystal formation did not occur. Calcium was not noted bound to the tissues, but iron, as was the case also with copper, was observed extensively on the surface of all the tissues.

The curves which compare the concentrations of copper in the snail emphasize the modifying influence of the living process and the effectiveness of the active transport system at keeping the system relatively free of copper even under the extremes of trauma and high copper concentration. What does find its way into the hemolymph of the snail is picked up by the p-cells.

The abrupt leveling off of the rise in snails without the living process at 16γ (Figure 1 b) probably results from depletion of carbon dioxide from the environment. The remaining even slope may represent the diffusion of carbon dioxide into the solution from the surrounding environment, perhaps augmented as the result of the binding of copper by the dead tissues.

The less steep rise during the first hour apparent in the curve of the living snails (Figure 1 a) apparently represents the formation of crystals on the surface and in the peripheral p-cells. Copper penetration then remains static until sometime after the 5th hour. The rise after the 5th hour would logically seem to be the result of the internal protein binding or the depositing of copper crystals in areas previously unavailable because of the resistance of the living process.

The inspection of the graphs offers little comfort to molluscicides. The resistance here observed against these high concentrations of copper used on snails having already been deprived of their first line of defense would seem to indicate their ability to withstand relatively long sieges of the lower concentrations normally used as molluscicides by snails possessing all their faculties.

It is not known how far the process of crystal formation can actually protect these snails from death but the combination of an impermeable shell with functional operculum, a considerable anaerobic capability and the enzymatic

deactivation of copper seem to make this prosobranch snail relatively immune to ordinary acute ionic attacks which might be lethal to less highly favored snails.

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